Chapter 10
Cellular Defense Mechanisms Following Nanomaterial Exposure: A Focus on Oxidative Stress and Cytotoxicity

Stephen J. Evans, Gareth J. Jenkins, Shareen H. Doak and Martin J. D. Clift

Abstract In response to the significant increase in nanotechnology over the last three decades, and the plethora of engineered nanomaterials (ENMs) now becoming available, understanding as to how nano-sized particles may impact upon human health has become a dominating area of research worldwide since the late 1990's (Stone et al. in Environmental Health Perspectives, 2017) [1]. Whilst approaches constantly adapt to the increasing number and variety of ENMs produced for a plethora of different applications, the quantity of alternative physico-chemical characteristics, a key factor in the potential hazard of ENMs (Bouwmeester et al. in Nanotoxicology 5:1–11, 2011) [2], is further increasing in number and type.

10.1 Introduction

In response to the significant increase in nanotechnology over the last three decades, and the plethora of engineered nanomaterials (ENMs) now becoming available, understanding as to how nano-sized particles may impact upon human health has become a dominating area of research worldwide since the late 1990s [1]. Whilst approaches constantly adapt to the increasing number and variety of ENMs produced for a plethora of different applications, the quantity of alternative physico-chemical characteristics, a key factor in the potential hazard of ENMs [2], is further increasing in number and type. Although it is well documented which characteristics influence ENM toxicity, the precise mechanism by which this observed toxicity occurs is not fully understood [3]. Despite this, as a result of increased laboratory-based investigations that have been conducted over the last three decades [1], a number of specific paradigms have been formulated in order to deduce and define the potential (human health) hazard posed by ENMs.

S. J. Evans · G. J. Jenkins · S. H. Doak · M. J. D. Clift (✉)
In Vitro Toxicology Group, Swansea University Medical School, Institute of Life Sciences, Singleton Park Campus, Swansea, Wales SA2 8PP, UK
e-mail: m.j.d.clift@swansea.ac.uk

© Springer Nature Switzerland AG 2019

P. Gehr and R. Zellner (eds.), *Biological Responses to Nanoscale Particles*, NanoScience and Technology, https://doi.org/10.1007/978-3-030-12461-8_10

10.2 Paradigms in Particle Toxicology

Of the three specific paradigms, also known as theory's, the main one is the 'oxidative stress paradigm', which is discussed in the latter paragraphs. However, while the potential for ENMs to cause oxidative stress has been the basis for increased research since the advent of nanoparticle toxicology in the early 1990s [4], two further paradigm's/theory's also exist; the fibre paradigm [5, 6], and the theory of genotoxicity [7, 8]. The latter is predominantly based upon the oxidative stress paradigm; however, it moves on from determining an inflammatory response to assess what the stimulation of oxidative and inflammatory mediators could induce to the biological system in regard to human health. The theory describes a two-tiered approach: (i) primary genotoxicity and (ii) secondary toxicity. In regard to primary genotoxicity, it is suggested that NPs can cause genotoxicity following direct exposure to the biological system. Secondary genotoxicity however describes, initially, the oxidative stress paradigm (in theory: NP exposure = ROS/reactive nitrogen species (RNS) production [also oxidative stress (oxidant/antioxidant imbalance)] = chronic inflammatory response), which causes genotoxicity and (possibly) subsequent tumour formation. Secondary genotoxicity, however, may not be caused by the NPs alone; it may also be caused via interaction of the biological system and the chemicals contained within the NPs. It is suggested that the NPs might be completely inert but are able to penetrate the cellular membrane, possibly locating within the nucleus. At this stage, due to the highly acidic pH, the chemicals present within the NPs could be released (such as Fe within Fe platinum NPs), causing a toxic response. This form of secondary toxicity has also been referred to as the "Trojan horse" effect [9]. It is also possible, however, in relation to this theory that the cells might undergo cell death and thus not induce genotoxicity and tumor formation.

As previously discussed in Clift and Rothen-Rutishauser [10], although both the oxidative stress paradigm and theory of genotoxicity can fit to any form or NP, they have predominantly been focused upon through the assessment of the biological response to spherical, crystalline, and non-fibrous NPs. However, since the portrayal that CNTs may cause "asbestos-like" effects in the lung [11], increased research has been performed using the theory of genotoxicity with fibrous NPs. While this is apt, specific, well-studied, and proposed paradigms already exist in regard to the biological effects following fibrous stimuli [6], including asbestos and glass wool; otherwise known as the fibre paradigm, with a specific set of rules, as detailed in Donaldson et al. [6]. Although there has been increased focus upon the fibre paradigm in regards to high aspect ratio nanomaterials (HARN), since the seminal paper of Poland and colleagues [11], and further intensity is being given towards the potential for ENMs to cause genotoxicity when considering a chronic, repeated and low-dose/concentration exposure to humans, the key toxicological paradigm associated with ENMs remains the 'oxidative stress paradigm' (Fig. 10.1).

Oxidative stress occurs when a greater number of oxidants than antioxidants are present within the cell, causing an oxidant/antioxidant imbalance. Increased oxidation can occur within cells, such as macrophages following activation. The activation

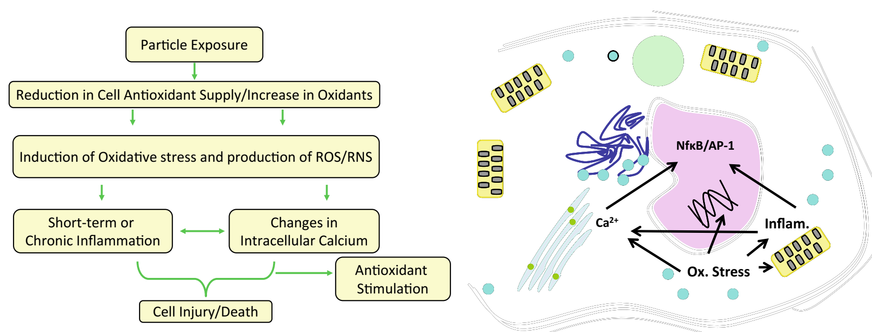


Fig. 10.1 Overview of the oxidative stress paradigm, as both a flow diagram (right) [10], and indicative within any mammalian cell (left). Both aspects highlight the foundation of the oxidative stress paradigm, where a ENM may induce an oxidative attack upon cells either independently, or cause inflammatory response which, both, can then impede cellular signaling pathways. This mechanistic toxic effect has then been known to initiate long-term detrimental effects upon cellular homeostasis (noted by the impact upon intracellular Ca^{2+} levels)

of macrophage cells can cause the generation of the superoxide anion, which is readily converted into the hydroxyl radical (•OH) via the influence of superoxide dismutase. The presence of the •OH, as well as the superoxide anion, which are examples of reactive oxygen species (ROS), can thus cause increased oxidation within the cell because these molecules possess unpaired electrons and are highly unstable. Additionally, ROS can be produced via—nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is the most common form of ROS found in cells, and is—usually produced when cells are performing the phagocytosis of xenobiotics. Therefore, this suggests that although cells purposefully clear hazardous particles from the tissue, the phagocytosing cells can unintentionally or intentionally produce ROS. In addition, the potential production of ROS following encapsulation of particles via phagocytosis further emphasizes the necessity to understand the specific uptake mechanism of NPs, in order to determine their potential route within the cell, and how their uptake may relate to their toxicity. As it is not possible to explore in detail the entry mechanisms of NPs into cells, the reader is referred to the comprehensive review of Unfried et al. [12].

The production of ROS and subsequent oxidative stress in cells can be extremely deleterious, causing a reduction in cell metabolic competence via a reduction in mitochondrial respiration as well as an increase in the production of (pro)inflammatory—mediators (i.e., cytokines and chemokines). The effects of ROS production and subsequent oxidative stress/inflammatory response can further be associated with diseases and illnesses such as pulmonary and cardiovascular diseases, including asthma, chronic obstructive pulmonary disease, as well as atherosclerosis and even tumor formation.

In a study by Stone et al. [1], the potential for NPs, specifically ufCB, to cause oxidative stress was assessed in A549 epithelial cells. Cells were measured for their

glutathione content to determine the level of oxidative stress present. Glutathione is the most abundant nonprotein thiol present inside most cells, and it is essential for maintaining cell viability by detoxification of pathogens as well as by regulating cell cycle and gene expression. A reduction in the glutathione content of cells, therefore, is known to indicate an oxidative stress environment. It is also known that glutathione can have a protective role following the onset of oxidative stress, due to its antioxidant gene characteristics. Antioxidant genes are common throughout cells, such as the c-*fos* gene. This gene is part of a series of growth- and differentiation–related genes that are expressed by cells in response to foreign materials. It is thought that antioxidant genes are "switched on" following NP stimulus, which can thus overwhelm the NP effect. Research into these effects, however, is limited and requires further investigation. In relation to the potential for NPs to induce oxidative stress, it was observed by Stone et al. [1] that following treatment of A549 epithelial cells with up to 0.78 μg mm^2 of ufCB and CB, the glutathione levels, as measured specifically in its reduced form (GSH), decreased after 2 h, with a significant decrease also found after 6-h exposure to ufCB, but not to CB. It was subsequently concluded by Stone et al. that ufCB is more potent at inducing oxidative stress than its larger counterpart, CB. Li et al. further studied the potential for NPs to induce oxidative stress in cells. In the study by Li et al., the effects of ambient (25–10 μm), fine (<2.5 μm), and uf (<0.1 μm) particles on RAW 264.7 macrophage cells and BEAS-2B epithelial cells were assessed. It was observed that NPs, in comparison to both ambient and fine particles, induced an increased cellular expression of heme-oxygenase–1 (HO-1) expression in each cell line, indicative of oxidative stress, as well as a decrease in intracellular glutathione levels. High levels of ROS production were also demonstrated following dithiothreitol (DTT) analysis. Further examination of the different particle types by transmission electron microscopy (TEM) showed the NPs, as well as a small amount of larger particles, to predominantly locate within mitochondria, causing subsequent major structural damage thought to contribute to increased oxidative stress (decrease in GSH) and toxicity previously observed. It was therefore concluded that the increased biological potency of NPs can be associated with the redox cycling of organic chemicals due to their increased ability to damage the mitochondria, causing ROS and oxidative stress. Recently, Xia et al. also showed ufCB, in comparison to TiO_2 and CB, to cause an increased production of ROS, as measured via the ROS quencher, furfuryl alcohol, as well as by assessment of the level of NADPH peroxidase with RAW 264.7 macrophage cells over a 4- and 16-h period. Subsequent analysis of the oxidative stress levels in these macrophage cells found depletion in GSH levels, as well as toxic oxidative stress after similar exposure periods. It was further illustrated that the toxic oxidative stress observed was specific to an injury to mitochondria due to increased cytosolic calcium (Ca^{2+}) production and uptake, causing structural damage to the organelle. Ca^{2+} is an essential and the most abundant mineral in the body. Maintaining normal Ca^{2+} levels (~155 ± 9 nM) within the cell, also known as Ca^{2+} homeostasis, is essential for cell viability. The finding by Xia et al. that an increased Ca^{2+} production (signaling) can occur in cells following exposure to NPs supports previous studies that have suggested the increase of cytosolic Ca^{2+} to be associated with the onset of increased ROS

production and subsequent oxidative stress. Stone et al. initially showed that ufCB exposure for 30 min to Mono Mac 6 (MM6) human macrophage cells induced a 1.6-fold increase in the resting cytosolic Ca^{2+}concentration, measured using the Ca^{2+} chelator Fura 2-AM in MM6 cells at a concentration of 66 μg/m, while no changes were observed following treatment of MM6 cells with CB. Subsequent investigation by Stone et al. examined the effects of CB, both fine and uf (33 μg/ml), and latex beads (64, 202, and 535 nm in diameter) (1 mg/ml) on MM6 cells and primary rat macrophages. Analysis of the latex beads showed a 2.3-fold increase in cytosolic Ca^{2+}concentration (as determined by using the Fura 2-AM molecule) in response to thapsigargin stimulation following treatment with the 64-nm latex beads; however, no effects on MM6 cell Ca^{2+} concentration were observed following treatment with either the 202- or 535-nm latex beads. In this study, thapsigargin was used to assess the viability of the cells via Ca^{2+} signaling. This chemical stimulant causes release of Ca^{2+} from the endoplasmic reticulum (ER); a loss of cell viability, via apoptosis, can be related to a loss of Ca^{2+} in the ER store and therefore a reduced Ca^{2+} response to thapsigargin stimulation. Similarly, Stone et al. showed a 2.6-fold increase in Ca^{2+} in BAL cells (>80% macrophages) following stimulation with thapsigargin and after exposure to ufCB, but not to CB. These latter findings support those previously reported by Stone et al. that ufCB can cause an increase in cytosolic Ca^{2+} concentration and further demonstrate that different macrophage types (MM6 and primary rat macrophages) can elicit similar responses following NP exposure. Further analysis by Stone et al. demonstrated that these effects were attenuated when the MM6 cells were pretreated with either the antioxidant mannitol or nacystelyn, suggesting that the increased cytosolic Ca^{2+} concentrations observed following NP exposure could be mediated via ROS and oxidative stress. Stone et al. performed further examination of the potential of all sizes of the latex beads to produce ROS, using the dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which when oxidized converts into 2′,7′-dichlorofluorescin (DCFH) and shows an increasing fluorescence intensity relative to an increase in ROS production. It was found that the NP latex beads (64 nm) were more potent in causing ROS, with the 202- and 535-nm latex beads showing no oxidative effects after 10 min. These findings were subsequently supported by Brown et al., who also measured cytosolic Ca^{2+} via the use of the fluorescent marker Fura 2-AM. It was reported that ufCB elicited a heightened cytosolic Ca^{2+} concentration in MM6 cells following treatment for 30 min. Subsequent analysis by Brown et al., which examined the effects of transition metals, specifically Fe in the form of Fe chloride ($FeCl_2$), using the transition metal chelator, Desferal, found that these metals had no effect on cytosolic Ca^{2+} concentration after 30 min of exposure. In addition, Brown et al. also investigated the inflammatory potential of the ufCB and CB particles as well as of the transition metals in vivo. It was observed that the ufCB particles, but not the CB particles, induced an increase in the number of PMNs present within the lungs of rats, with a significant increase in the number of neutrophils found within the BAL fluid after 24-h exposure. No inflammatory effects were found with $FeCl_2$. Following inductively coupled plasma–mass spectrometry, it was observed that the $FeCl_2$ particles were detectable in ng mg^{-1} concentrations within ufCB particles. It was subsequently concluded that

ufCB does elicit an increase in cytosolic Ca^{2+} concentration, in support of Stone et al., and that ufCB particles induce an inflammatory response from cells, which was suggested to be independent of the transitional metal content of these NPs. The observation by Brown et al. that ufCB can cause increased inflammation supports the suggestion that ROS production, oxidative stress, and altered Ca^{2+} signaling caused by these NPs can cause an activation of transcription pathways, via a cascade of events within the cell, including both the nuclear factor–kappa B (NF-κB) and activator protein-1 (AP-1) pathways. Activation of these pathways was subsequently proposed to result in an escalated inflammatory response, with an increase in proinflammatory gene expression and proinflammatory mediator production, such as the cytokines IL-8 and TNF-α. Subsequent analysis by Brown et al. further supported this hypothesis, demonstrating that ufCB particles, and not CB particles, cause an increase in resting cytosolic Ca^{2+} concentration (as assessed by using Fura 2-AM) in rat alveolar macrophages. Similar effects were also found on treatment of rat alveolar macrophages in a dose–response relationship (12.5–50 μg/ml) following thapsigargin stimulation. It is also worth noting that these effects are similar to the findings of Stone et al. and Brown et al., further demonstrating the consistency between different forms of macrophage cells in assessing the toxicity associated with NP exposure. Additional study by Brown et al. also showed that these effects were decreased following addition of antioxidants (Trolox and nacystelyn), further supporting the findings of Stone et al., who concluded that the increased Ca^{2+} signaling observed in macrophage cells after NP exposure was mediated via ROS. As it was suggested, by Donaldson et al., that subsequent inflammation could occur due to the activation of specific transcription pathways (such as NF-κB and AP-1) following the altered Ca^{2+} signaling caused by ROS and oxidative stress of NP-treated cells, Brown et al. further studied the effects of ufCB on both NF-κB and AP-1 transcription pathways. Investigation of the NF-κB pathway showed ufCB (100 μg/ml) treated human monocyte cells to show increased fluorescence of the sub-units of NF-κB, p50, and p65 over a 4-h period compared with untreated monocyte cells. Additional analysis of the AP-1 pathway showed no significant increase in the intensity of the AP-1 protein following treatment with ufCB at 200 μg/ml, after 4 h in rat alveolar macrophages. The findings relative to both these pathways were found to be attenuated following the addition of antioxidants, further supporting the suggestion that these events are mediated via ROS production. Subsequent analysis of the inflammatory potential of ufCB in rat alveolar macrophages found the production of the proinflammatory cytokine TNF-α to be dose dependent (25–200 μg/ml) after 4-h exposure. It was subsequently concluded by Brown et al. that uf particles can exert proinflammatory effects by altering Ca^{2+} signaling, activating transcription factors and causing the production of proinflammatory cytokines via ROS-mediated mechanism, thus supporting the proposed oxidative stress paradigm. Although the findings of these studies suggest that the toxicity observed following exposure to NPs is relative to increases in the production of ROS and subsequent oxidative stress, as well as an escalation in Ca^{2+} signaling and inflammation within cells, the oxidative paradigm is only a hypothesis, and further research is required to fully understand the mechanisms of NP toxicity and how they may relate to the many new and different types of NPs being manufactured.

It is prudent to note that the oxidative stress paradigm is flexible. In the previously discussed literature, it is evident that oxidative stress induces an inflammatory response which affects cell signaling. This sequence, while correct, is not always true for NPs. Any resultant effects can be initiated following the onset of oxidative stress following NP exposure occur either in the presence or absence of a state of oxidative stress.

10.3 Cellular Defense Mechanisms in Mammalian Cells

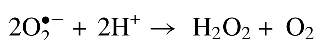
Whilst oxidative stress has been well studied in terms of the mechanics of the toxicological response to ENMs, it is a common misconception that it is a negative aspect within mammalian cells, and only associated with a hazard effect response. Yet, in terms of the cellular defence of any cell type within the human body, there are a number of able defense mechanisms, of which oxidative stress is one. Such defense mechanisms include specific active (and passive) uptake mechanisms (please refer to Conner and Schmid [13] since it is not the absolute intention of this chapter is not to provide an overview of the different uptake mechanisms possible by any mammalian cell type). Yet, a side-effect of this the two major forms of 'cell-eating', or scenario is also the inflammatory response, which is another defense mechanism of the human body to any foreign body invasion (including ENMs). Yet, of all the defense mechanisms that mammalian cells have, it is their ability to engage the redox action that creates an imbalance between the cells antioxidant defence system, and the oxidants present in the cell/tissue.

10.4 Oxidative Stress, Antioxidants and Reactive Oxygen Species

As noted above, the major mechanism by which nanomaterials (NMs) are considered to induce cellular toxicity is via oxidative stress, which refers to a cellular redox imbalance as a result of increased intracellular highly Reactive Oxygen Species (ROS). The term ROS encompasses a number of molecules and free radicals derived from oxygen including primary ROS–H_2O_2, $O2^-$ and secondary ROS–OH• [14]. During normal cellular function ROS are produced as by-products of metabolism. For example, a one electron gain by the oxygen molecule (O_2) results in the formation of the superoxide free ion $O_2^{\bullet}$. This reduction happens frequently during numerous biological processes such as the electron transfer chain within the mitochondria; as several components of complexes I, II and III express thermodynamic properties required for the reduction of O_2 to O_2^- [15]. Other cellular source of O_2^- include the microsomal transfer chain via NADPH-cytochrome P_{450} and NADH-cytochrome b_5 reductase activities, the respiratory burst action of phagocytic cells, peroxisomal

beta-oxidation and Fenton reactions [16]. At low levels ROS may act as 'redox messengers' in intracellular signalling [17]. This is achieved by the activation of Redox sensitive transcription factors include AP-1, p53 and NF-κB which regulate pro-inflammatory cytokine expression, cell differentiation and apoptosis [18]. This signalling maybe utilised during the initiation of an inflammatory response with in a tissue for example.

Due the fact that ROS are a natural cellular occurrence due to normal processes, a homeostasis is maintained by a series of antioxidant proteins. The main class of this antioxidants is superoxide dismutases (SOD) including Cu–Zn–SOD (SOD1) and Mn–SOD (SOD2). Both SOD1 and SOD2 catalyse the conversion of O_2• to the less reactive H_2O_2 which can subsequently be converted to H_2O by catalyse and glutathione (GSH) [19]:



$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

The role of antioxidants is critical to maintaining cellular health, if an imbalance occurs between the levels of ROS and antioxidants, indiscriminate damage may be inflicted on a range of biological molecules. This include lipid peroxidation where ROS attack polyunsaturated fatty acids within the cell membrane, this results in the formation of a peroxyl-fatty acid radical and a subsequent chain reaction of membrane damage [20]. Lipid peroxidation can ultimately lead to impaired cellular functioning and cell rupture. Furthermore oxidative damage to the mitochondrial membrane can result in electron chain dysfunction and subsequently cell death [21]. ROS can also promote protein oxidation resulting in fragmentation at amino acid residues, protein cross links and oxidation of the amino acid chains resulting in loss of function [22]. The ability of ROS to cause protein damage has the potential to impact a multitude of cellular functions in addition to the risk of a build-up of malformed protein within the cell. In addition to protein oxidation and lipid peroxidation a key risk is ROS-induced DNA damage which is typified by single and double stranded DNA breaks, base modification (e.g. DNA adducted formation and DNA cross linkage) [23].

10.5 NMs and Oxidative Stress

A number of NMs have been shown to be inducers of oxidative stress, in particular metal oxide nanoparticles which may release ions capable of inducing the formation of the highly reactive hydroxyl radical ($^{\bullet}OH$) by conversion of H_2O_2 by Fenton chemistry.

H_2O_2 is not reactive as it has no unpaired electrons but it is however a mediator in the formation of secondary ROS in the form of hydroxyl radicals (•OH). This •OH formation can be initiated via transition metal ion promoted Fenton chemistry [24, 25]

$$\mathbf{M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + {}^{\bullet}OH + OH^-}$$

M represents transition metal.

Transition metal based NM's such as iron, copper, nickel, cobalt, and zinc may therefore release ions that can take part in the Fenton reaction promoting an increase in intracellular •OH formation. This free radical presents a significant risk for DNA damage as •OH is capable of attacking the DNA backbone and nucleotide bases promoting the formation of DNA lesions. More than 20 oxidative base lesions have been identified, the most notable being 8-hydroxygyanine (8-OH-dG) which frequently miss-pairs with thymine resulting double stranded breaks and point mutations [26].

A number of studies have identified transition metal based NM's as inducers of oxidative stress. For example, copper oxide promoted increased micronucleus formation in the Neuro-2A cell line as a resulted of oxidative damage measured by the formation of malondialedhyde (MDA) [27]. Moreover, significant MDA formation has been exhibited in the brains of Wister rats following treatment with gold (Au) nanoparticles (NPs) [28]. Perhaps the mostly widely studied transition metal NM is silver (Ag) due to it antimicrobial properties. Indeed, Ag NPs have been shown to induce ROS formation in lung epithelial cells (A549) as measured by the 2′-7′-Dichlorodihydrofluorescein diacetate (DCFDA) assay [29]. Similarly, when tested in HepG2 cells Ag NPs promoted increased ROS production (quantified by DCFDA) promoting downstream double stranded DNA breaks [30].

NM oxidative stress potential not limited to those comprised of transition metals, a number of NMs have been shown to catalyse ROS production at their surface in aqueous suspension including silica and carbon nanotubes [31]. This is likely due to immobilised free bonds of the atoms located on the NM surface. Quartz NPs for instance have been associated with the generation of ROS due to the presence of surface bound SiO• and SiO_2• [32]. Furthermore, the quantum confinement effect of quantum dots modulates their ability to accept and donate electric charge and potentially enable them to catalyse ROS formation [33].

10.6 NM Induced Immune Response and Oxidative Stress

If a NM is capable of promoting an immune response in vivo this may result in the formation of ROS by the cellular components of the immune system. NMs have indeed been shown to be capable of triggering ROS production in activated phagocytes (macrophages and neutrophils) in the form of a NADPH mediated respiratory burst [34–36]. If this respiratory burst is maintained downstream oxidative damage may be promoted in other cell types within the NM exposed tissue. ROS themselves are in fact mediators in the activation and recruitment of other immune cells, by promoting inflammatory cytokine production via activation of the transcriptional regulatory factor NF-κB. A vicious circle of chronic inflammation inducing downstream genotoxicity is therefore a possible scenario upon NM exposure [7].

10.7 ROS and Cytotoxicity

Due to the ability of ROS to mediate redox sensitive transcription factors its excessive presence in with in the cell can cause activation of apoptosis. This can be initiated by the upregulation of the tumour suppressor protein p53 which one cell stress is low can induce cell cycle arrest and DNA repair [37]. At high levels of cell stress however p53 can down regulate pro-survival factors, upregulate apoptotic factors and induction of the caspase cascade [38]. Due to the association of the upregulation of TNFα and ROS there is also evidence of linkage between ROS and apoptosis initiated by the extrinsic pathway [39].

10.8 Summary

The field of nanoparticle toxicology is a complex discipline that incorporates a plethora of different disciplines. It allows for the gaining of novel understanding towards an aspect that is vital regarding human long-term health effects. To date, there has been limited indication that nanomaterials are able to affect long-term human health, but this is due to a lack of research into this area and also the model systems to study it. Instead acute effects have been focussed upon, that have shown that commonly, realistic exposure concentrations/doses used in studies indicate that cellular machinery is often impeded, most notably by mechanisms associated with an oxidative stress response. Whilst oxidative stress is normal, it occurs within every organ/tissue/cell routinely, excess oxidative stress (commonly caused through reactive oxygen/nitrogen species) is a negative cellular response that can have both hazardous acute and chronic effects (e.g. inflammatory response), and so is essential to maintain in regards to the ENM-cell interaction.

Acknowledgements The authors would like to acknowledge all members of the In Vitro Toxicology group who contribute to the exhaustive scientific discussions.

References

1. Stone, V., Miller, M.R., Clift, M.J D., Elder, A., Mills, N.L., Moller, P., Schins, R.P.F., Vogel, U., Kreyling, W.G., Jensen, K.A., Kuhlbusch, T.A.J., Schwarze, P.E., Hoet, P., Pietroiusti, A., De Vizcaya-Ruiz, A., Baeza-Squiban, A., Tran, L., Cassee, F.R.: Nanomaterials vs ambient ultrafine particles: an opportunity to exchange toxicology knowledge. Environ. Health Perspect. (2017) http://dx.doi.org/10.1289/EHP424
2. Bouwmeester, H., Lynch, I., Marvin, H.J.P., Dawson, K.A., Berges, M., Braguer, D., Byrne, H.J., Casey, A., Chambers, G., Clift, M.J.D., Elia, G., Fernandes, T.F., Fjellsbø, L.B., Hatto, P., Juillerat, L., Klein, C., Kreyling, W.G., Nickel, C., Riediker, M., Stone, V.: Minimal analytical characterisation of engineered nanomaterials needed for hazard assessment in biological matrices. Nanotoxicology **5**, 1–11 (2011)

3. Clift, M.J.D., Gehr, P. Rothen-Rutishauser, B.: In vitro testing for Nanotoxicology: a valid alternative? Arch. Toxicol. **85**, 723–731 (2011)
4. Ferin, J., Oberdorster, G., Penney, D.P.: Pulmonary retention of ultrafine and fine particles in rats. Am. J. Respir. Cell Mol. Biol. **6**, 535–542 (1992)
5. Donaldson, K., Tran, C.L.: An introduction to the short-term toxicology of respirable industrial fibres. Mutat. Res./Fundamen. Mol. Mechan. Mutagen. **553**, 5–9 (2004)
6. Donaldson, K., Murphy, F.A., Duffin, R., Poland, C.A.: Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part Fibre Toxicol. **7**, 5 (2010)
7. Evans, S.J., Clift, M.J., Singh, N., de Oliveira Mallia, J., Burgum, M., Wills, J.W., Wilkinson, T.S., Jenkins, G.J., Doak, S.H.: Critical review of the current and future challenges associated with advanced in vitro systems towards the study of nanoparticle (secondary) genotoxicity. Mutagenesis **4** (2017)
8. Schins, R.P., Knaapen, A.M.: Genotoxicity of poorly soluble particles. Inhal. Toxicol. **19**, 189–198 (2007)
9. Limbach, L.K., Li, Y., Grass, R.N., Brunner, T.J., Hintermann, M.A., Muller, M., Gunther, D., Stark, W.J.: Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. Environ. Sci. Technol. **39**, 9370–9376 (2005)
10. Clift, M.J.D., Rothen-Rutishauser, B.: Studying the oxidative stress paradigm in vitro: a theoretical and practical perspective. In: Armstrong D., Bharali, D. (eds.) Oxidative Stress and Nanotechnology vol. 1082, pp. 115–133 (2013)
11. Poland, C.A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W.A.H., Seaton, A., Stone, V., Brown, S., MacNee, W., Donaldson, K.: Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotech **3**, 423–428 (2008)
12. Unfried, K., Albrecht, C., Klotz, L.O., von Mikecz, A., Grether-Beck, S., Schins, R.P.F.: Cellular responses to nanoparticles: target structures and mechanisms. Nanotox **1**, 1–20 (2007)
13. Conner, S.D., Schmid, S.L.: Regulated portals of entry into the cell. Nature **422**, 37–44 (2003)
14. Gamaley, I.A., Klyubin, I.V.: Roles of reactive oxygen species: signaling and regulation of cellular functions. Int. Rev. Cytol. **188**, 203–255 (1999)
15. Cadenas, E., Boveris, A., Ragan, C.I., Stoppani, A.O.M.: Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. Arch. Biochem. Biophys. **180**, 248–257 (1977)
16. Poljsak, B., Šuput, D., Milisav, I.: Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid. Med. Cell. Longev **11** (2013)
17. Circu, M.L., Aw, T.Y.: Reactive oxygen species, cellular redox systems and apoptosis. Free Radic. Biol. Med. **48**, 749–762 (2010)
18. Burton, G.J., Jauniaux, E.: Oxidative stress. Best Pract. Res. Clin. Obstet. Gynaecol. **25**, 287–299 (2011)
19. Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., Dong, W.: ROS and ROS-mediated cellular signaling. Oxid. Med. Cell. Longev. **2016**, 18 (2016)
20. Vasilaki, A.T., McMillan, D.C.: Lipid peroxidation. In: Schwab, M. (ed.) Encyclopedia of Cancer. Springer, Berlin, Heidelberg (2011)
21. Manke, A., Wang, L., Rojanasakul, Y.: Mechanisms of nanoparticle-induced oxidative stress and toxicity. Biomed. Res. Int. **2013**, 15 (2013)
22. Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R.: Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta **329**, 23–38 (2003)
23. Singh, N., Manshian, B., Jenkins, G.J., Griffiths, S.M., Williams, P.M., Maffeis, T.G., Wright, C.J., Doak, S.H.: NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials **30**, 3891–3914 (2009)
24. Valko, M., Izakovic, M., Mazur, M., Rhodes, C., Telser, J.: Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell Biochem. **266** (2004)

25. Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., Mazur, M.: Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. **160**, 1–40 (2006)
26. Cooke, M., Evans, M., Dizdrogla, M., Lunec, J.: Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. **17** (2003)
27. Perreault, F., Pedroso Melegari, S., Henning da Costa, C., de Oliveira Franco Rossetto, A.L., Popovic, R., Gerson Matias, W.: Genotoxic effects of copper oxide nanoparticles in Neuro 2A cell cultures. Sci. Total Environ. **441,** 117–124 (2012)
28. Siddiqi, N.J., Abdelhalim, M.A.K., El-Ansary, A.K., Alhomida, A.S., Ong, W.: Identification of potential biomarkers of gold nanoparticle toxicity in rat brains. J. Neuroinflamm. **9**, 1 (2012)
29. Foldbjerg, R., Dang, D.A., Autrup, H.: Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. Arch. Toxicol. **85**, 743–750 (2011)
30. Kim, S., Choi, J.E., Choi, J., Chung, K.H., Park, K., Yi, J., Ryu, D.Y.: Oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma cells. Toxicol. In Vitro **23**, 1076–1084 (2009)
31. Magdolenova, Z., Collins, A., Kumar, A., Dhawan, A., Stone, V., Dusinska, M.: Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology **8**, 233–278 (2014)
32. Huang, Y.-W., Wu, C.-H., Aronstam, R.S.: Toxicity of transition metal oxide nanoparticles: recent insights from in vitro studies. Materials **3**, 4842 (2010)
33. Abdal dayem, A., Hossain, M.K., Lee, S.B., Kim, K., Saha, S.K., Yang, G.-M., Choi, H.Y., Cho, S.-G.: The role of reactive oxygen species (ros) in the biological activities of metallic nanoparticles. Int. J. Mol. Sci. **18**, 120 (2017)
34. Sun, S., Wang, Q., Giang, A., Cheng, C., Soo, C., Wang, C.-Y., Liau, L., Chiu, R.: Knockdown of CypA inhibits interleukin-8 (IL-8) and IL-8-mediated proliferation and tumor growth of glioblastoma cells through down-regulated NF-kB. J. Neurooncol. **101** (2011)
35. Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., Schiestl, R.H.: Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Can. Res. **69**, 8784–8789 (2009)
36. Tulinska, J., Kazimirova, A., Kuricova, M., Barancokova, M., Liskova, A., Neubauerova, E., Drlickova, M., Ciampor, F., Vavra, I., Bilanicova, D., Pojana, G., Staruchova, M., Horvathova, M., Jahnova, E., Volkovova, K., Bartusova, M., Cagalinec, M., Dusinska, M.: Immunotoxicity and genotoxicity testing of PLGA-PEO nanoparticles in human blood cell model. Nanotoxicology **1**, 33–43 (2015)
37. Kaminskyy, V.O., Zhivotovsky, B.: Free radicals in cross talk between autophagy and apoptosis. Antioxid. Redox. Signal. **21**, 86–102 (2014)
38. Redza-Dutordoir, M., Averill-Bates, D.A.: Activation of apoptosis signalling pathways by reactive oxygen species. Biochimica et Biophysica Acta (BBA)—Mol. Cell Res. **1863**, 2977–2992 (2016)
39. Vandenabeele, P., Galluzzi, L., Berghe, T.V., Kroemer, G.: Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat. Rev. Mol. Cell Biol. **11**, 700 (2010)